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VULGARONE B, THE ANTIFUNGAL CONSTITUENT IN THE STEAM-DISTILLED FRACTION OF *Artemisia douglasiana*

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Abstract—Antifungal activity of the steam distilled essential oil fraction of *Artemisia douglasiana* was detected by bioautography on silica gel TLC plates against three *Colletotrichum* spp. The active principle was isolated by bioassay-directed fractionation using column chromatography followed by crystallization and was characterized as vulgarone B by ^{1}H and ^{13}C NMR and GC-MS. Antifungal activity of vulgarone B was further evaluated using 96-well microtiter assay against *Colletotrichum acutatum, C. fragariae, C. gloeosporioides*, and *Botrytis cinerea*. In addition, the antifungal activity of vulgarone B and verbenone, and their corresponding alcohols was tested by bioautography and microtiter assay. Structure–activity studies revealed that the α, β-unsaturated carbonyl functionality is a prerequisite for the antifungal activity of these mono and sesquiterpene ketones. This is the first report of antifungal activity of vulgarone B. The yield of essential oil from *A. douglasiana* is about 0.6–0.8% by weight of the dry material, including plant stems.

Key Words—Antifungal activity, vulgarone B, bioautography, *Artemisia douglasiana*, microtiter assay, α, β-unsaturated ketones, *Colletotrichum acutatum*, *Colletotrichum fragariae*, *Colletotrichum gloeosporioides*, *Botrytis cinerea.*

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INTRODUCTION

Filamentous fungi of the genus *Colletotrichum* and its telemorph *Glomerella* are major plant pathogens worldwide (Farr et al., 1989). *Colletotrichum* species often cause typical symptoms of anthracnose, a disease characterized by sunken necrotic lesions usually bounded by a red margin (Freeman et al., 1998). Anthracnose diseases of strawberry (*Fragaria x ananassa* Duch.) are serious problems for fruit and plant production in many areas of the world (Mass and Palm, 1997; Mass, 1998). The pathogens, *Colletotrichum acutatum* J. H. Simmonds, *C. gloeosporioides* (Penz.) Penz., and *C. fragariae* A. N. Brooks, can occur singly or in combination, and can infect flowers, fruits, leaves, petioles, stolons, and crowns of the strawberries (Howard et al.,1992; Smith, 1998a,b,c).

Botrytis fruit rot is among the most destructive diseases of strawberry fruits. Fruits are infected just before harvest, especially when the crop experiences persistent wetness. The causal fungus, *Botrytis cinerea* Pers. Fr. also affects blossom blight and may infect strawberry leaves and petioles (Sutton, 1998). Fungicidal sprays have been widely used for the control of *Botrytis* fruit rot, but in some areas *B. cinerea* has become resistant to many fungicides. Resistance to the most commonly used classes of fungicides, the benzimidazoles and dicarboximides, has seriously compromised the effectiveness of these fungicides. Few fungicides are now available for effective control of *Botrytis* diseases. New approaches for disease control have become necessary as the effectiveness and availability of commercial fungicides decrease.

Among different procedures used to minimize crop losses, chemical control of causative fungi remains the most common and effective method. A number of synthetic fungicides are used for this purpose (Vyas, 1988). Owing to emergence of resistant strains of certain pathogenic fungi, some currently used fungicides are less effective. There is a need for more selective, safe, and environment friendly fungicides. Natural products offer a pool of structurally diverse antifungal agents. Following natural product leads, more potent compounds and a better understanding of the mode of action of the fungicides can be achieved.

In our continuing search for antifungal compounds of natural origin against the fungi that are responsible for anthracnose disease of strawberries, the steam-distilled oil fraction of the aerial parts of *Artemisia douglasiana* Besser, a shrub from Oregon was investigated. *Artemisia* belongs to the family Asteraceae. Many members of the Asteraceae have been shown to possess diverse biological activities (Wat et al., 1980, 1981; Hudson et al., 1991, 1993). *Artemisia* species are often considered weeds in many parts of the world. Many weeds are ecologically important and contain bioactive compounds such as allelopathic and antifungal constituents in order to survive in the ecosystem. In this paper we report the isolation and identification of the antifungal compound from the steam distillate of *A. douglasiana* and the structural requirements for the antifungal activity.

METHODS AND MATERIALS

Instrumentation. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer operating at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR. GC-MS analysis was carried out on HP5790 MSD spectrometer (Hewlett Packard, USA) equipped with 5890 GC using a DB-1 column 20-m $\times$ 0.2-mm, 0.18-μm film thickness (J & W Scientific Inc., Folsom, CA). The oven was temperature programmed from 60°C (5 min) to 280°C (20 min) at 5°C/min, total run: 69 min. The carrier gas was helium.

Materials. Steam distilled oil of aerial parts of *A. douglasiana*, a bluish colored oil, was provided by Aromagen, Albany, Oregon, USA. The steam distillation was performed on dry aerial parts of the plant grown in Albany, Oregon, USA, from 1998 harvest. The oil sample was stored in the freezer at -25°C until use. Verbenone and verbenol standards and $LiAlH_4$ (LAH) were purchased from Aldrich Chemical. Fungicide standards thiophanate methyl, captan, and thiabendazole were purchased from Chem Service, West Chester, PA, USA. Thin layer chromatography (TLC) was performed on silica gel GF plates, 0.25-mm thickness (Analtech Inc., Newark, DE, USA) using 20% ethyl acetate in hexane. Anisaldehyde spray reagent, iodine vapor, and UV light were used for the detection of compounds on TLC plates. Column chromatography was performed on Kieselgel 60 (particle size 0.063–0.2 mm) silica gel (Merck, Germany) with hexane and methylene chloride in increasing amounts. Solvents were reagent grade and were used without further purification.

Pathogen Production and Inoculum Preparation. Pathogen production and inoculum preparation for *B. cinerea*, *C. gloeosporioides*, *C. fragariae*, and *C. acutatum* were carried out according to the published procedures (Wedge and Kuhajek, 1998; Wedge et al., 2000). Conidial concentrations were determined photometrically (Wedge and Kuhajek, 1998) from a standard curve and suspensions were then adjusted with sterile distilled water to a concentration of 1.0×10^6 conidia/ml.

Bioautography. Bioautography using *Colletotrichum* spp. was used to identify the antifungal activity according to the previously published method (Wedge et al., 2000). Crude steam distillate of *A. douglasiana* was separated on silica TLC plates using 20% ethyl acetate and hexane, and then air-dried. Each plate was subsequently sprayed with a spore suspension (10^6 spores/ml) of the fungal species of interest and then incubated in a moist chamber for 3 days at 24°C with a 12 hr photoperiod under 60 ± 5 μmol/m^2/sec light. The regions where the antifungal constituents were present on the TLC plate were detected as clear zones, in a dark background on the TLC plate due to the inhibition of fungal growth.

Microbioassay. The steam distillate and the purified active compound isolated was further evaluated for quantitative antifungal activity using a 96-well format according to previously published methods (Wedge and Kuhajek, 1998; Wedge et al., 2000). Thiabendazole and captan with two different modes of action

were used as standards in this experiment. Thiophanate methyl was used as the standard in evaluating the activity of the crude steam distillate. Steam distillate was tested at 20, 50, and 100 μg/ml, whereas purified compound and standards were tested at 0.3, 3.0, and 30 μM concentrations against *B. cinerea*, *C. fragariae*, *C. gloeosporioides*, and *C. acutatum* in 96-well microtiter plates (Nunc Micro Well, untreated; Roskilde, Denmark). The steam distillate was tested at 20, 100, and 500 μg/ml. Microtiter plates were covered with a plastic lid and incubated in a growth chamber at 24 $\pm$ 1°C and 12 hr photoperiod under 60 $\pm$ 5 μmol/m^2/sec. Fungal growth was monitored photometrically by measuring absorbance at 620 nm at 24, 48, and 72 hr. Mean absorbance values and standard errors were used to evaluate fungal growth at 48 hr. Analysis of variance of the Least Square Means for percent inhibition of each fungus at each dose of test compound relative to the untreated controls was used to evaluate fungal growth inhibition. The experiment used a repeated measures design and was repeated once. Data presented were pooled from two independent experiments.

Isolation of the Antifungal Constituent. The steam distilled oil (5 g) was column chromatographed on silica gel column (30-cm $\times$ 35-mm id) using hexane and increasing amounts of CH_2Cl_2(0–80% CH_2Cl_2 in hexane, with hexane 2l, 20% with 31, 40% with 41, and 80% with 21). Fractions of 250 ml were collected, concentrated at 40°C, and the fractions with similar TLC profiles were combined to afford 22 fractions. Each fraction was tested on TLC bioautography to select the antifungal active fractions. Fractions that showed antifungal activity were pooled according to TLC profile and further purified by silica gel column chromatography (20-cm $\times$ 25-mm id) using CH_2Cl_2 in hexane. The antifungal compound, vulgarone B (**1**), was eluted in fractions 6–14 with hexane–CH_2Cl_2 (1:1). This was crystallized with 1% CH_2Cl_2 in hexane at 4°C to afford 580 mg of white crystalline compound. ^{1}H NMR: δ ($CDCl_3$) 5.71 (1H, br s, 2-H), 2.73 (1H, d, J = 6.4 Hz, 11-H), 2.52 (1H, d, J = 6.4 Hz, 4-H), 2.11 (1H, s, 5-H), 1.98 (3H, br s, 15-H_3), 1.68 (2H, m, 9-H_2) 1.62 (2H, m, 8-H_2), 1.39 (2H, m, 7-H_2), 0.92 (6H, s, 13-H_3, 14-H_3), 0.83 (3H, s, 12-H_3). ^{13}C NMR: δ ($CDCl_3$), 204.9 (C-1), 172.6 (C-3), 122.5 (C-2), 66.8 (C-5), 57.9 (C-11), 55.1 (C-10), 49.9 (C-4), 41.5 (C-9), 38.5 (C-7), 33.8 (C-6), 27.7 (C-12), 27.2 (C-13), 24.6 (C-14), 23.3 (C-15), 21.3 (C-8). $M^+ = 218$. The identity was confirmed by NMR and $[\alpha]_D$ with those reported in the literature (Uchio et al., 1977).

Reduction of Vulgarone B. To an ice cold suspension of LAH (30 mg) in anhydrous ether (15 ml) vulgarone B (**1**) (20 mg) in 10 ml of anhydrous ether was added and the mixture was stirred for 45 min at room temperature. The reaction was quenched by pouring the mixture into 0.5% HCl in H_2O (100 ml). The mixture was extracted with ether (3 $\times$ 150 ml), the ether layer was dried over anhydrous Na_2SO_4 and evaporated in vacuo to yield a white crystalline product (19 mg) that was further purified by TLC using 10% ethyl acetate–hexane. The allylic alcohol vulgarol B (**2**) with its β-orientation was obtained as white crystals and

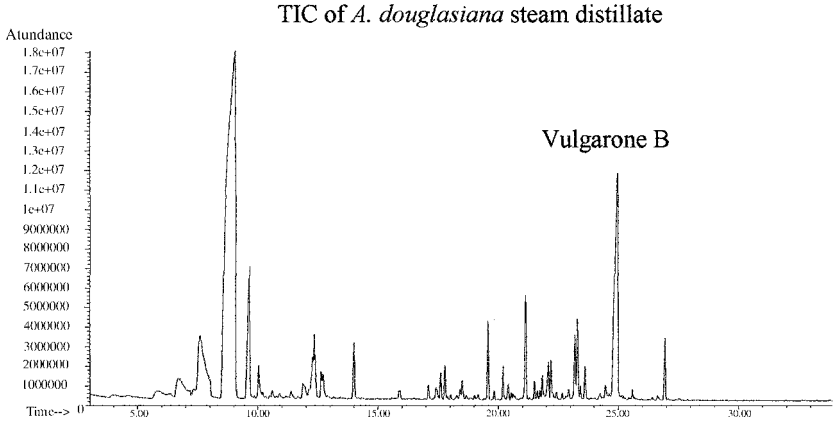

FIG. 1. A sample gas chromatogram of *A. douglasiana* steam distillate.

was identified by comparison of its NMR data and $[\alpha]_D$ with those reported in literature (Uchio et al., 1977). ^{1}H NMR: δ ($CDCl_3$) 5.32 (1H, br s, 2-H), 4.38 (1H, br s, 1-H), 2.35 (1H, m, 11-H), 2.03 (1H, d, $J = 6.3$ Hz, 4-H), 1.92 (1H, s, 5-H), 1.71 (3H, br s, 15-H_3), 1.68–1.53 (4H, m, 8-H_2, 9-H_2), 1.33 (2H, m, 7-H_2), 1.00 (3H, s, 14-H_3), 0.86 (3H, s, 12-H_3), 0.80 (3H, s, 13-H_3). ^{13}C NMR: δ ($CDCl_3$), 150.0 (C-3), 120.2 (C-2), 75.4 (C-1), 61.7 (C-5), 47.4 (C-11), 47.1 (C-4), 41.3 (C-9), 40.4 (C-10), 38.5 (C-7), 32.6 (C-6), 28.1 (C-12), 27.1 (C-13), 24.8 (C-14), 22.4 (C-15), 21.5 (C-8). $M^+ = 220$.

GC-MS Analysis of Vulgarone B. The concentration of vulgarone B in the crude oil sample of *A. douglasiana* was determined by GC-MS analysis using selective ion mode. A calibration curve was obtained using standard solutions of pure vulgarone B and plotted using linear regression ($R^2 = 0.99$). The concentration of vulgarone B in the crude oil was *ca.* 11% by weight (Figure 1).

RESULTS AND DISCUSSION

Preliminary screening of the steam distilled oil of *A. douglasiana* by bioautography on silica gel TLC plates revealed the presence of antifungal agents inhibitory to *C. acutatum*, *C. fragariae*, and *C. gloeosporioides* (Figure 2). Microbioassay of the crude steam distillate also indicated inhibitory activity against *C. acutatum*, and *B. cinerea* where thiophanate-methyl was used as the fungicide standard (Table 1). Bioassay guided fractionation of the steam distilled oil by column chromatography facilitated the isolation of the antifungal compound that was identified by ^{1}H and ^{13}C NMR and GC-MS as vulgarone B (**1**), a sesquiterpene ketone, previously been isolated from *Chrysanthemum vulgare* (Uchio et al., 1977). The concentration of

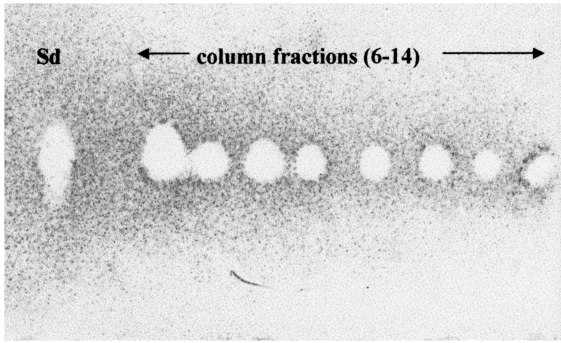

FIG. 2. Bioautography of *Artemisia douglasiana* steam distillate (Sd) and silica gel column fractions. The plate was sprayed with spores of *Colletotrichum fragariae.* Clear zones demonstrate area of fungal growth inhibition and indicate the presence of antifungal constituents.

vulgarone B in the steam-distilled oil of *A. douglasiana* was determined by GC-MS to be *ca.* 11%.

In order to establish some structure–activity relationships, verbenone (**3**), vulgarone B (**1**), and the corresponding allylic alcohols, verbenol (**4**) and vulgarol B (**2**), obtained by $LiAlH_4$ reduction of (**1**) (Figure 3), were assessed by bioautography. Only compounds possessing an α, β-unsaturated carbonyl group had antifungal activity (Figure 4). Hence, it is evident that this functional group is essential for the antifungal activity of vulgarone B and verbenone. Therefore, we postulate that one of the possible modes of action of these antifungal compounds may be due to their roles as Michael-type acceptors for biological nucleophiles (Ahn and sok, 1996). Evaluation of the quantitative antifungal activity of vulgarone

TABLE 1. PERCENT INHIBITION OF FUNGAL SPECIES BY STEAM-DISTILLED OIL OF *Artemisia douglasiana*

Organism	Sample	Mean ± SD (48 hr fungal growth)[a]		
		20 μg/ml	100 μg/ml	500 μg/ml
B. cinerea	Thiophanate-methyl[b]	24.0 ± 10.3	5.1 ± 3.6	−36.7 ± 36.5
	A. douglasiana steam distillate	35.2 ± 5.7	−26.1 ± 35.2	−98.5 ± 10.3
C. acutatum	Thiophanate-methyl	18.2 ± 50.2	−20.3 ± 37.6	−86.3 ± 7.9
	A. douglasiana steam distillate	23.2 ± 23.2	−44.5 ± 11.6	−95.5 ± 6.3

[a] Negative values represent the strength of fungal growth inhibition and positive values represent growth stimulation relative to the untreated controls. Data are pooled from two different experiments repeated in time.

[b] Commercial fungicide standard.

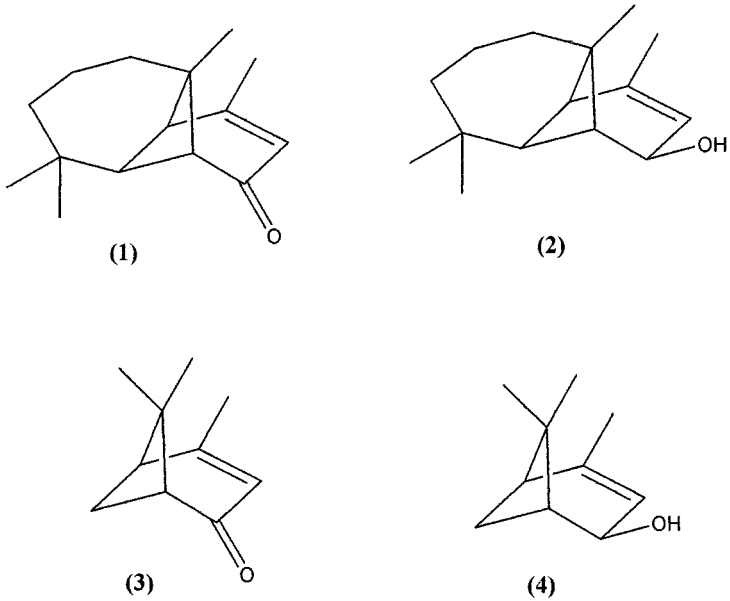

FIG. 3. Structures of the compounds: (1) Vulgarone B, (2) Vulgarol B, (3) Verbenone, (4) Verbenol.

B and verbenone was carried out using 96-well microbioassay system in comparison with commercial fungicide standards thiabendazole and captan according to published methods (Wedge and Kuhajek, 1998; Wedge et al., 2000). Growth inhibition results indicated that vulgarone B and verbenone had some selectivity toward *B. cinerea,* (Figure 5) but had marginal activity at concentrations $\leq$30 μM (data not

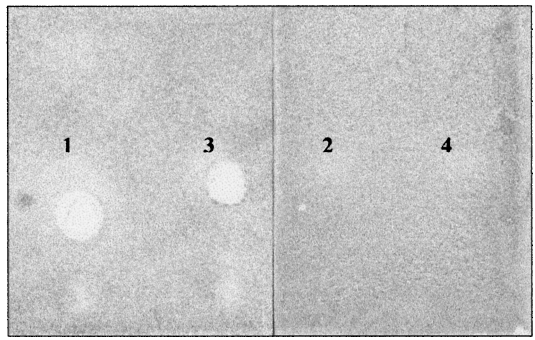

FIG. 4. Bioautography of vulgarone B (**1**), verbenone (**3**), and their corresponding alcohols **2** and **4**. Only **1** and **3** showed antifungal activity.The plate was sprayed with spores of *Colletotrichum fragariae.*

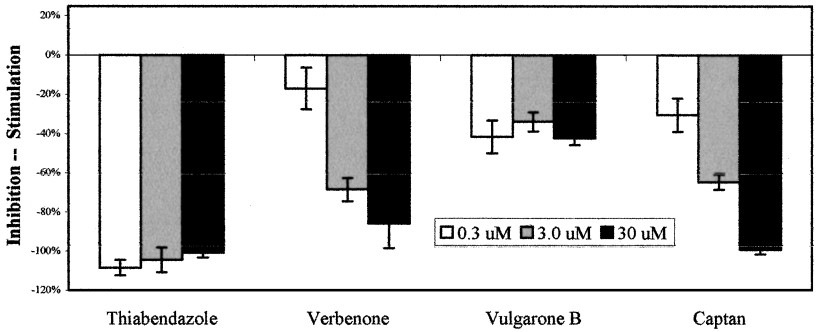

FIG. 5. Growth inhibition of *Botrytis cinerea* at 48 hr using a 96-well microbioassay format in response to verbenone (**3**) and vulgarone B (**1**) and the commercial fungicide standards thiabendazole and captan. Means for percent growth inhibition were pooled from two experiments replicated in time.

shown) against *C. gloeosporioides*, *C. fragariae*, *C. acutatum*, and *F. oxysporum*. At 30 μM concentrations, vulgarone B showed *ca*. 40% inhibition of *B. cinerea* growth and 20% inhibition of *C. fragariae* growth. Verbenone showed much greater antifungal activity against *B. cinerea* (*ca*. 80% growth inhibition) and lower activity against *C. fragariae* (20% growth inhibition). At 30 μM concentrations, the commercial fungicides thiabendazole and captan showed *ca*. 90% inhibition of *B. cinerea* growth. These results indicate that vulgarone B and verbenone are moderately active against *B. cinerea* and marginally active against the *Colletotrichum* species that we tested. While the antifungal mode of action of vulgarone B and verbenone is yet to be determined, this is the first report of antifungal activity of vulgarone B and verbenone against *Colletotrichum* and *Botrytis* species. Future greenhouse studies will determine the potential commercial applications for these compounds to control fungal diseases.

In summary, there are numerous reports in the literature about the antifungal activity of essential oils but reports on isolation and identification of the individual components that are responsible for the biological activity are limited (Pattnaik et al., 1997; Meepagala et al., 2002; Vila et al., 2002). This research has demonstrated the antifungal activity of steam-distilled oil of *A. douglasiana* against *B. cinerea*, and marginal activity against *C. acutatum*, *C. fragariae*, and *C. gloeosporioides*. The antifungal component of *A. douglasiana* was isolated and identified as vulgarone B (**1**). This is the first report demonstrating the antifungal activity of vulgarone B to agriculturally important plant pathogenic fungi. Moreover, we have also shown the importance of the α, β-unsaturated carbonyl group for the antifungal activity of this class of compounds. Further investigation of the mode of action and use of this compound as a postharvest fumigant shall be performed.

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